

Applicants: Nalam Madhusudhana Rao and Priyamvada Acharya  
Serial No.: 10/768,951  
Filed : January 29, 2004  
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**In the Specification**

Please replace the Sequence Listing in the subject application with the Sequence Listing attached hereto as **Exhibit A**.

On page 9, replace the paragraph that begins on line 6 and ends on line 16 with the following new paragraph:

-- In the present invention a method of directed evolution were applied to the lipase gene (~~Gene~~Seq ID No.123) to isolate protein variants of the original sequence which possess increased thermostable properties. The methodology relies initially on the ability to create random variations in the original gene sequence and express the corresponding proteins in the bacteria, E.coli. The produced variants of the original sequence would have altered sequence, hence altered properties. The variants, at a proteins level , would be tested for their thermostability and those sequences which demonstrate improved thermostability would be subjected to the next round of random mutagenesis and screening. Thus by sequential accumulation of the mutants and subsequent pooling of the mutations the thermostability of lipase was improved by 200-fold at high temperature. High temperature range includes temperature ranges from 50-90 C. --

On page 9, replace the paragraph that begins on line 28 and ends on line 4 of page 10 with the following new paragraph:

-- The naturally occurring lipase from *Bacillus lipase* has the amino acid sequence of 1-181 as given in the SEQ ID No.1 (See also SEQ ID No. 23 for corresponding gene sequence). It was discovered that the amino acid substitutions at positions 68, 71, 114, 120, 132, 144, 147 and 166 were found to be important

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for the thermostability of the lipase. In accordance with the present investigation, it was further discovered that the substitutions at positions 114, 132 and 166 are suited for increasing the stability of the proteins. Any of the innumerable combinations of substitutions possible at each of these positions with the other 19 amino acids would be favourable for the thermostability.--

On page 10, replace the paragraph that begins on line 14 and ends on line 18 with the following new paragraph:

-- Accordingly, the main embodiment of the present invention relates to the novel thermostable, organic solvent resistant and high pH tolerant lipase gene variants having SEQ ID No. 2 of molecular wt 19443 (See also SEQ ID No. 24 for corresponding gene sequence), SEQ ID No. 3 of molecular wt 19515 (See also SEQ ID No. 25 for corresponding gene sequence), SEQ ID No. 4 of molecular wt 19456.9 (See also SEQ ID No. 26 for corresponding gene sequence), SEQ ID No.5 of molecular wt.19487 (See also SEQ ID No. 27 for corresponding gene sequence), ~~and~~ and SEQ ID No.6 of molecular wt. 19470.9 (See also SEQ ID No. 28 for corresponding gene sequence). --

On page 10, replace the paragraph that begins on line 19 and ends on line 24 with the following new paragraph:

-- Another embodiment of the present invention relates to an expression system for novel thermostable, organic solvent resistant and high pH tolerant lipase gene variants said expression system comprising of having SEQ ID No. 2 of molecular wt 19443 (See also SEQ ID No. 24 for corresponding gene sequence), SEQ ID No. 3 of molecular wt 19515 (See also SEQ ID

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No. 25 for corresponding gene sequence), SEQ ID No. 4 of molecular wt 19456.9 (See also SEQ ID No. 26 for corresponding gene sequence), SEQ ID No.5 of molecular wt. 19487 (See also SEQ ID No. 27 for corresponding gene sequence) and SEQ ID No.6 of molecular wt 19470.9 (See also SEQ ID No. 28 for corresponding gene sequence) present in the vector pJO290.--

On page 10, replace the paragraph that begins on line 25 and ends on line 30 with the following new paragraph:

-- Still another embodiment of the present invention relates to the a method of preparing an expression system of novel thermostable, organic solvent resistant and high pH tolerant lipase gene variants having SEQ ID No. 2 of molecular wt 19443 (See also SEQ ID No. 24 for corresponding gene sequence), SEQ ID No. 3 of molecular wt 19515 (See also SEQ ID No. 25 for corresponding gene sequence), SEQ ID No. 4 of molecular wt 19456.9 (See also SEQ ID No. 26 for corresponding gene sequence), SEQ ID No.5 of molecular wt. 19487 (See also SEQ ID No. 27 for corresponding gene sequence) and SEQ ID No.6 of molecular wt 19470.9 (See also SEQ ID No. 28 for corresponding gene sequence), said method comprising the steps of: --

On page 23, replace the paragraph that begins on line 27 and ends on line 6 of page 24 with the following new paragraph:

-- The mutant Gene sequence~~5~~ 27 was created from the clone 2-8G10 and wt by using the unique restriction site Hae II at position 910 of the lipase gene. The genes coding for the two proteins were amplified by PCR using the T7 promoter and terminator primers. The PCR products were purified by gel extraction and digested with Hae II and Nde I. The upper and

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lower bands correspond to the C-terminal and N-terminal regions of the protein, respectively. The upper band from clone 2-8G10 and the lower one from the wild-type protein were eluted. The higher molecular weight fragment was digested with BamH I and purified. A three point ligation containing the Nde I- Hae II fragment (from the wt), the Hae II-BamH I fragment (from 2-8G10) and pET-21b cut with Nde I and Hae II was set up, the ligation mix transformed into DH5 $\alpha$  and the positives selected. The sequence of the gene was confirmed by DNA sequencing.--

On page 24, replace the paragraph that begins on line 7 and ends on line 11 with the following new paragraph:

-- The mutant Gene sequence--6\_28 (triple mutant) was created by site-directed mutagenesis on the Gene sequence 5 template using the mutagenic primer PROLF: 5'- GGC AAG GCG CCT CCG GGA ACA GAT-3' to incorporate a codon change CTT  $\rightarrow$  CCT that led to L114P change in the amino acid sequence. The sequences of all the genes were confirmed by automated DNA sequencing.--